Separation of isomeric metabolites of carbamazepine by liquid chromatography and high resolution accurate mass

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The environmental concerns about the presence of excreted pharmaceuticals in wastewater are well documented [1] and the introduction of high resolution mass spectrometry (HRMS) such as Time of Flight and Orbitrap instruments has aided their detection. Although HRMS is a highly specific technique, interferences can occur especially in complex matrixes. This paper describes how some of the problems encountered were overcome when analysing wastewater samples for carbamazepine (CBZ) and its metabolites using a Thermo Scientific Orbitrap Q Exactive HRMS instrument [2].

CBZ is a widely prescribed drug used to treat epilepsy and neuropathic pain and it is known to be a persistent environmental pollutant which is not broken down during wastewater treatment.

Most drugs are metabolised prior to excretion, however detection of these metabolites in wastewater is only beginning to be routinely tested. CBZ is excreted mainly as the trans-10,11-dihydro-10,11-dihydroxycarbamazepine (trans-CBZdiOH) metabolite but also forms an epoxide metabolite, carbamazepine epoxide (CBZEP), which gives negative side effects in humans [3] and is toxic in the environment. There are also five mono-hydroxy metabolites of CBZ having the same precursor ion exact mass as CBZEP and because they are all structurally similar yield the same product ion in highest abundance on fragmentation. Oxcarbazepine (OxCBZ) is a replacement for CBZ which was developed as it does not metabolise to the toxic CBZEP but, it also has the same molecular formula as CBZEP and the monohydroxy metabolites. This makes it difficult to distinguish between the different metabolites even using HRMS and requires careful interpretation of the data and good chromatography to ensure the mono-hydroxy metabolites are separated from CBZEP and a false high concentration is not reported. Another new drug substitute for CBZ is eslicarbazepine (EsliCBZ) which was also included in this study.

A summary of the chemical structures, precursor ion and molecular formula of the analytes included in this study are shown in *Table 1*.

Table 1. Chemical structures and precursor ions in this study.

Molecular Formula = $C_{15}H_{12}N_2O_2$ Accurate mass precursor ion ES$^+$: m/z 253.0977 [M+H]$^+$	
10,11-epoxy-10,11-dihydrocarbamazepine (CBZEP)	10-monohydroxy carbamazepine (10-CBZmonohydroxy)
monohydroxy carbamazepine (CBZmonohydroxy)	Oxcarbazepine
Carbamazepine $C_{15}H_{12}N_2O$ Accurate mass precursor ion ES$^+$: m/z 237.1028 [M+H]$^+$	Cis-10,11-dihydro-10,11-dihydroxycarbamazepine $C_{15}H_{14}N_2O_3$ Accurate mass precursor ion ES$^+$: m/z 271.1083 [M+H]$^+$
Eslicarbazepine $C_{17}H_{16}N_2O_3$ Accurate mass precursor ion ES$^+$: m/z 297.1239 [M+H]$^+$	Trans-10,11-dihydro-10,11-dihydroxycarbamazepine $C_{15}H_{14}N_2O_3$ Accurate mass precursor ion ES$^+$: m/z 271.1083 [M+H]$^+$
10,11-dihydro-10-hydroxycarbamazepine(CBZ-10hydroxydihydro) $C_{15}H_{14}N_2O_2$ Accurate mass precursor ion ES$^+$: m/z 255.1133 [M+H]$^+$	

The mass spectrometer used for the analysis was a Thermo Scientific Q-Exactive Orbitrap mass spectrometer, fitted with a Dionex Ultimate 3000 RS Pump, Dionex Ultimate 3000 RS Autosampler (Temperature controlled at 10°C) and Dionex Ultimate 3000 RS Column Compartment (Temperature controlled at 30°C).

The software was Chromeleon®, Xcalibur™ and Tracefinder™.

Ionisation was by electrospray in positive ionisation mode with a spray voltage of 3.5 kV. The sheath and auxiliary gas were 45 and 10 arbitrary units respectively and the capillary and auxillary temperature were both 300°C. The range of the full MS-SIM experiment was 50 – 750 *m/z*, the target ions were specified in the targeted MS^2 method with an isolation window of 4 *m/z*. For both experiments the mass resolution was 35000.

The chromatography column was a Waters Atlantis® dC18 chromatography column 150 × 2.1 mm, particle size 3 µm.

Mobile phase A was methanol and B was 0.1% formic acid in Ultra-pure water. The LC gradient started at 99% B for 2 minutes then to 70% B over 3 minutes and maintained at that for 11 minutes. The composition of B was dropped to 1% over 1 minute and maintained at that for 3 minutes. Finally returning to 99% B over 1 minute and re-equilibrated for 9 minutes. The flow rate was 0.2 mL/minute and injection volume of 10 µL.

Solutions containing CBZ, cis-CBZdiOH, trans-CBZdiOH, CBZEP, OxCBZ, EsliCBZ and CBZ-10hydoxydihydro at a concentration of 1000 ng/mL were first injected separately to determine the retention time of each analyte. These were then injected as a mixture.

On processing the mixture three peaks were observed at the specific transition of 237.11 → 194.0963 for CBZ (*Figure 1A*). This was a synthetic mixture and the solution should not contain any other drugs with this specific transition or precursor ion and the loss of specificity was initially concerning. The interference at 7.9 minutes corresponded to EsliCBZ and at 12.5 minutes 10,11-dihydro-10-hydroxycarbamazepine with transitions 297.12 → 194.0963 and 255.11 → 194.0963 respectively. Although these have the same product ion the precursor ion is very different for each.

Similar contamination was observed for CBZEP at 9.5 minutes, this time the interferences had retention time of 6.4 and 7.2 minutes which were consistent for cis- and trans-CBZdiOH.

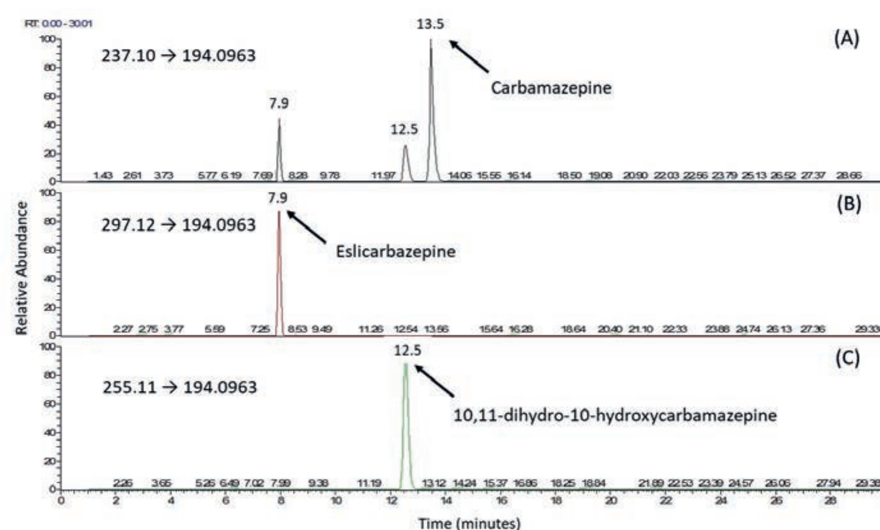

Figure 1. Chromatograms of carbamazepine, eslicarbazepine and 10,11-dihydo-10-hydroxycarbamazepine.

Closer inspection suggested this may be due to fragmentation in the ion-source. Although electrospray is a soft ionisation technique some analytes can break down in the ion source [4].

Experiments were conducted to confirm the additional peaks were due to in-source fragmentation. A solution of EsliCBZ was infused directly into the ion source at a flow of 10 µL/min.

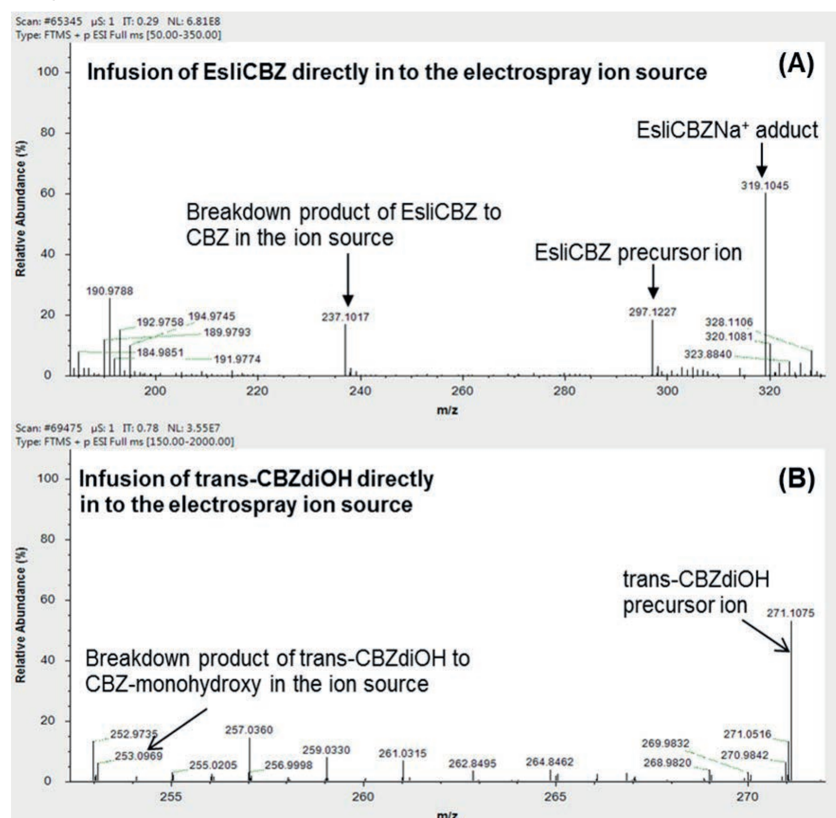

Figure 2. In-source fragmentation of EsliCBZ (A) and trans-CBZdiOH (B) introduced into the ion source by infusion at a concentration of 1000ng/mL.

Infusion of a solution of EsliCBZ into the ion source (*Figure 2A*) produced the expected precursor ion for EsliCBZ at *m/z* 297.1227, a sodium adduct *m/z* 319.1045 and an in-source breakdown product of *m/z* 237.1017 consistent with CBZ. During chromatography EsliCBZ elutes from the column and on entering the ion source partially breaks down to CBZ. This means at the retention time for EsliCBZ there are two precursor ions present, EsliCBZ at *m/z* 297.1227 and the break down ion at *m/z* 237.1017. Both of these will travel through the mass spectrometer and produce their respective product ions in the collision cell.

A similar breakdown was observed when trans-CBZdiOH was infused into the ion-source.

Infusion of a solution of trans-CBZdiOH into the ion source (*Figure 2B*) produced the expected precursor ion for trans-CBZdiOH *m/z* 271.1075 and a breakdown ion *m/z* 253.0969 consistent with CBZEP and CBZmonohydroxy. The infusion experiments were carried out under normal ion-source conditions and no fragmentation energy was applied in the ion source.

These two infusion experiments prove some conversion can take place in the ion source. Where conversion has occurred the analytes are well separated by retention time. However, the monohydroxy metabolites have yet to be tested and this adds to the complexity of analysing CBZ and its metabolites. Therefore, robust chromatography and careful interpretation of the precursor and product ion data is required to ensure the correct analyte was selected and accurately measured.

Due to the lack of mono-hydroxy standards, a sample of wastewater was used to determine the retention times for all the metabolites. The excellent sensitivity and resolution of the instrument aided the ability to inject the samples directly on to the liquid chromatography mass spectrometry system with only filtration of the samples prior to analysis, this was limited to a few samples. This would ensure no analytes were missed due to poor recovery during a sample clean-up or concentration step.

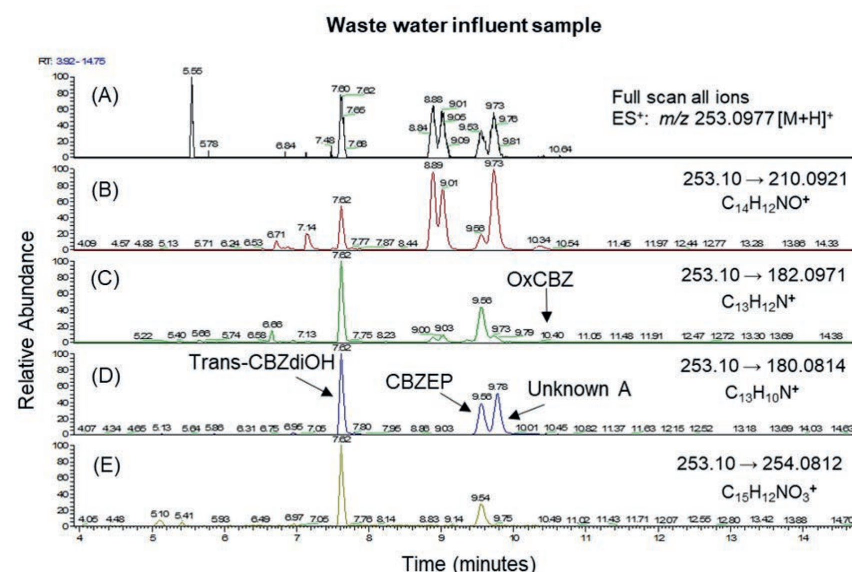

Figure 3. Ions at m/z 253.0977 in a wastewater sample.

Analysis of wastewater samples using the developed LC method determined multiple peaks with the transition *m/z* 253.10 → 210.0921 for some samples. The 3 main product ions from 253.10 were *m/z* 180.0814, 182.0971 and 210.0921. From the CBZEP standard another product ion at *m/z* 254.0812 was observed. *Figure 3B* demonstrates the transition *m/z* 253.10 → 210.0921 is less specific, especially for CBZEP but would be the ion of choice for the monohydroxy metabolites. The peaks eluting before trans-CBZdiOH (7.6 minutes) are thought to be the glucuronide metabolites. With careful selection a more specific transition (*m/z* 253.10 → 182.0971) was determined for the epoxide which further distinguished it from the interferences. Using the improved chromatography method and the specific transition afforded a good quantitation method for CBZEP. Unknown 'A' had a precursor ion *m/z* 255.1133 and was not 10,11-dihydro-10-hydroxycarbamazepine as both had different retention times. The isolation window for the tMS^2 experiment was changed to 2 *m/z* which removed this interference.

From the CBZEP standard, the transitions 253.10 → 182.0971 and 253.10 → 254.0812, were specific to CBZEP and using these product ions CBZEP was separated from the other analytes (*Figure 3 C and E*). The product ion with *m/z* 254.0812 is unusual in that it has a higher molecular weight than the precursor ion. The accurate mass fitted for a molecular formula of $C_{15}H_{12}NO_3^+$. This can be explained by the epoxide ring opening to a di-hydroxy and the loss of NH_2 (*Figure 4B*).

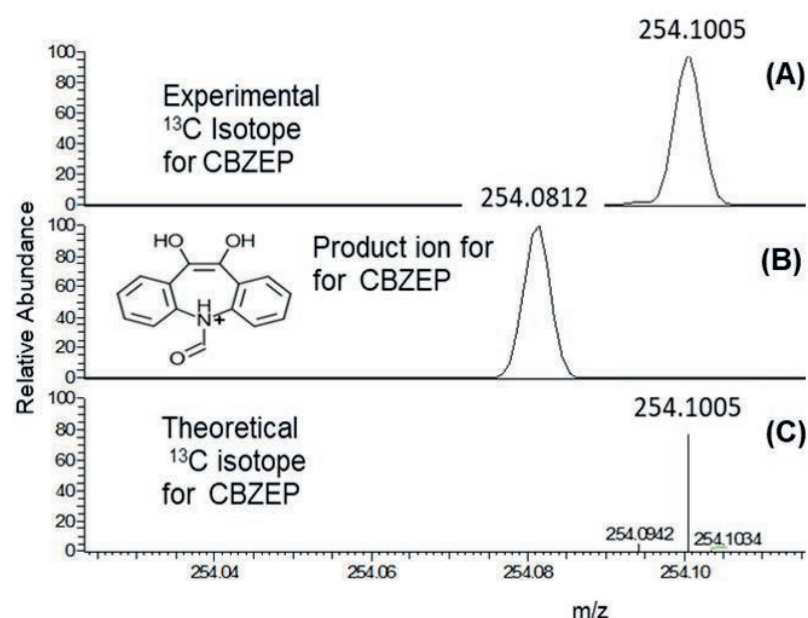

Figure 4. Comparison of experimental and theoretical pattern of CBZEP product ion (253.10 → 254.0809) and the extracted ion chromatogram of the ^{13}C isotope of CBZEP = 254.1002.

It was necessary to confirm the product ion (*m/z* 254.0812) was not the ^{13}C isotope of CBZEP. The theoretical ^{13}C isotope for CBZEP is in the inset (*Figure 4C*). Comparing the experimental ion and the theoretical isotope pattern for CBZEP confirms this is a product ion of CBZEP (*m/z* 254.0810) and not the ^{13}C isotope (*m/z* 254.1005). Using an instrument with lesser resolution the product ion for CBZEP (*m/z* 254.0821) could not be used as it would not be possible to distinguish it from the ^{13}C isotope (*m/z* 254.1015)

HRMS is extremely sensitive and selective however, robust chromatography is still essential for complex mixtures and care has still to be taken interpreting the HRMS data to prevent interferences and false positives.

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